THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 24, Issue of December 25, pp. 15127-15136, 1982
Printed in U.S.A.

A Comparison of Rat Myosin from Fast and Slow Skeletal Muscle and the Effect of Disuse*

(Received for publication, July 14, 1981)

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Certain enzymatic and structural features of myosin, purified from rat skeletal muscles representative of the fast twitch glycolytic (type IIb), the fast twitch oxidative (type IIa), and the slow twitch oxidative (type I) fiber, were determined and the results were compared with the measured contractile properties. Good correlation was found between the shortening velocities and Ca^{2+}-activated ATPase activity for each fiber type. Fast twitch white (type IIb) and mixed fast twitch red (type IIa/IIb) muscles could not be distinguished physiologically and showed three identical isomyosins (FM1, FM2, and FM3) by nondenaturing electrophoresis. The relative abundance of fast twitch light chains (LC) in rat white muscle (type IIb) is comparable with other mammalian fast twitch muscles, except for the reduced amount of LC_{3f} in the rat. The low level of LC_{3f} in the rat is corroborated by correlation between light chain distribution and the ratio of fast myosin isomyosins. Fast twitch red type IIa and fast twitch white type IIb muscles have similar myosin ATPase activities and maximal shortening velocity, but differ in terms of isomyosin profile and in the percentage of light chains and light chain stoichiometry.

Short term hind limb immobilization caused prolongation of contraction time and one-half relaxation time in the fast twitch muscles and a reduction of these contractile properties in slow twitch soleus. Furthermore, the increased maximum shortening velocity in the immobilized soleus could be correlated with increased Ca^{2+}-ATPase, but no change was observed in the enzymatic activity of the fast twitch muscles. No alteration in light chain distribution with disuse was observed in any of the fiber types. The myosin from slow twitch soleus could be distinguished from fast twitch myosins on the basis of the pattern of peptides generated by proteolysis of the heavy chains. Six weeks of hind limb immobilization resulted in both an increased ATPase activity and an altered heavy chain primary structure in the slow twitch soleus muscle.

Skeletal muscle function is reflected in the physiological and biochemical properties of the contributing fiber types (1-4). Fast twitch white fibers (type IIb) have relatively short isometric twitch contraction times, high maximal shortening velocities (V_{max}), and high myosin ATPase activities and fatigue rapidly. Fast twitch red fibers (type IIa) also demonstrate relatively short isometric twitch contraction times and high V_{max} and myosin ATPase activity, but are more resistant to fatigue. Slow twitch oxidative fibers (type I) show a prolonged isometric contraction time, a low V_{max} and low specific activity of myosin ATPase and a high resistance to fatigue.

The myosin molecule of vertebrate striated muscle has been well characterized and contains two heavy chains and two pairs of light chains (5-9). The catalytic site for myosin ATPase and the actin-binding site appear to reside solely on the myosin heavy chains (10-12), but the role that the light chains play in modulating the actomyosin system is not well understood. The ability of chicken fast myosin S-1 to retain both high ATPase activity and actin binding, when stripped of the alkali light chains (12), has been interpreted in terms of the subunits stabilizing the conformation of the myosin heavy chains. Although vertebrate skeletal fast twitch muscle myosin heavy chain exhibits the same ATPase activity as myosin S-1 (13), the fact that chemical modification of the single cysteine residues, located not at the catalytic site but on each alkali light chain of rabbit fast myosin, inhibits both actin binding and ATP hydrolysis (14) suggests that these light chains may participate in regulation of the actomyosin system. Using physiological conditions, recent work has demonstrated a functional role for the Ca^{2+}-binding light chain, LC_2.[1] The extraction of LC_2 from skinned skeletal muscle fiber with EDTA was found to be temperature dependent, and loss of one-third of the total LC_2 markedly decreased the maximum velocity of shortening with little effect on isometric tension (15). A 50% dissociation of LC_2 from rabbit myosin, treated with EDTA, has been reported to cause a 15-20% reduction in actomyosin ATPase activity (16). In each case, subsequent reassociation of the light chain reversed the effects of EDTA treatment.

It would appear logical to anticipate that correspondence between the molecular properties of myosin and the physiology of contraction, may best be approached by comparing the biochemical and mechanical responses of muscles, homogeneous for a particular fiber type, in an experimental regimen designed to alter muscle function. In this respect, it is known that the pattern of muscular activity affects both the physiological and the biochemical properties of skeletal muscle (17-19). Also, chronic electrical stimulation favors the transformation of fast to slow muscle (17, 20) and inactivity promotes the conversion of slow muscle to fast (21, 22).

We have approached this question by determining mechanical properties and comparing them with certain structural

* This work was supported by Grant AM 22037 from the National Institutes of Health and by National Aeronautics and Space Administration Grant NAS9-15711. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a Dr. William M. Scholl Predoctoral Fellowship for research in biomedical sciences.

§ Supported by National Institutes of Health Research Career Development Award AM 00810. To whom reprint requests should be addressed.

[1] The abbreviations used are: LC, light chain, EDL, extensor digitorum longus; SVL, superficial region of the vastus lateralis; DVL, deep red portion of the vastus lateralis; SDS, sodium dodecyl sulfate; FM, fast twitch myosin; SM, slow twitch myosin.

and enzymatic features of myosin from control and 6-week immobilized rats. Specifically, this study reports an integrated physiological and biochemical analysis of muscles containing primarily fast white (type IIb), fast red (type IIa), or slow red (type I) fibers, from control and 6-week immobilized rat hind limb preparations. Since sample size was limited by the casting procedure, a relatively simple method was developed for extracting myosin. The myosin light chain content was determined by one-dimensional and two-dimensional gel electrophoresis and compared with the ATPase activities. Results showed good correlation between the shortening velocities and the Ca^{2+}-activated ATPase activity of each muscle type. Fast twitch white (type IIb) and the mixed fast twitch red (type IIa and type IIb) muscles could not be distinguished physiologically. Six weeks of hindlimb immobilization caused the slow twitch soleus (type I) to become more like a fast twitch muscle in terms of contractile properties and myosin Ca^{2+}-ATPase, without alteration in light chain pattern. Limited proteolysis demonstrated that the 1-D peptide pattern of immobilized soleus heavy chain was different from the control soleus and fast red myosin appeared to differ from fast white in terms of heavy chain structure, but the peptide pattern of these fast twitch muscles was unaffected by disuse.

MATERIALS AND METHODS

Tissues—Sprague-Dawley 5–6-month-old female rats provided hind limb muscles of histochemically identified fiber type. The vastus lateralis and EDL provided fast twitch fibers. The superficial region of the vastus lateralis is 100% pure fast white type IIb fibers (23); the deep red portion of the vastus lateralis is 70% fast red type IIa fibers and 30% slow red-type I fibers (23). The EDL, used for physiological studies, is 60% fast red-type IIa fibers and 40% fast white-type IIb fibers (24). The soleus provided slow red muscle, being composed of 85% slow twitch-type I fibers and 15% fast red-type IIa fibers (24).

Casting—As an experimental model, muscle disuse and inactivity were produced by simple immobilization of rat hind limbs using plaster casts (25). Immobilization by casting maintains nerve and blood supply intact and causes muscular atrophy without the complications of contracture introduced by tenotomy (26).

Myofibril Preparation—Selected muscles (SVL, DVL, EDL, and soleus) were rapidly removed from the hind limbs of parallel groups of control or 6-week immobilized rats. The muscle tissue was cleaned of fat, fascia, and tendons and thoroughly minced. The mince was blended in 0.1 M KCl, 5 mM $MgCl_2$, 5 mM ethylene glycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 1 mM Na pyrophosphate, 25 mM imidazole, pH 7.0, using a Sorvall Omni-Mixer. The myofibrils were pelleted by centrifugation at 800 × *g* for 10 min and washed three times in an imidazole buffer (25 mM, pH 7.0) by centrifugation and resuspension. All procedures were performed at 0 °C, in order to ensure relaxed myofibrils. The three times-washed myofibrils were either frozen overnight at −70 °C prior to myosin extraction or immediately solubilized in isoelectric focusing buffer containing 9.5 M urea (1:2, v/v) and another 1 g of solid urea was added per 3 ml of total volume, prior to freezing. Myofibrillar proteins were resolved by isoelectric focusing, followed by two-dimensional gel electrophoresis, exactly as described by Whalen *et al.* (27).

Myosin Extraction—The regimen necessary for maintenance of rats in hind limb plaster casts, combined with the resulting muscular atrophy, limited the sample size available, especially of the small soleus muscle. Myosin, prepared from slow twitch cat and rabbit soleus by conventional procedures, has been reported as unacceptably contaminated by other muscle proteins (7, 20). We have, prepared myosin by a procedure that incorporates two rounds of actomyosin dissociation, each followed by high speed centrifugation to remove actin (20) and a final ammonium sulfate precipitation step (28). In this manner, myosin was prepared of a purity suitable for the electrophoretic and enzymatic characterization of the three fiber types.

Myofibrils were first prepared in order to maximize the yield of myosin (7). Since proteolytic degradation was considered a problem during the extraction of cat soleus myosin (20), all extraction buffers contained 0.1 mM phenylmethanesulfonyl fluoride (final concentration). The presence of this protease inhibitor also enhances the resolution of contractile proteins by gel electrophoresis (29). The washed myofibrils were resuspended in buffer I (0.3 M KCl, 5 mM $MgCl_2$, 5 mM ATP, 2 mM sodium pyrophosphate, 0.5 mM dithiothreitol, 0.15 M KH_2PO_4, pH 6.6). The actomyosin was dissociated by gently stirring the myofibrils on ice for 20 min. The extracted myofibrils were centrifuged at 165,000 × *g* for 4 h to pellet actin (20). The myosin was precipitated from the supernatant by dialysis overnight against low salt buffer (5 mM KH_2PO_4, 20 mM KCl, adjusted to pH 6.3). The precipitated myosin was collected by low speed centrifugation and resuspended in buffer II (0.6 M KCl, 5 mM $MgCl_2$, 5 mM ATP, 0.5 mM dithiothreitol, 2 mM sodium pyrophosphate, 0.15 M KH_2PO_4, adjusted to pH 6.6). The ATP dissociation, high speed centrifugation, and overnight dialysis were repeated as above. The precipitated myosin was washed three times with low salt buffer to remove nucleotides. The final myosin pellet was resuspended in buffer III (0.5 M KCl, 5 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothreitol, 1 mM EDTA, 32.5 mM K_2HPO_4, 17.5 mM KH_2PO_4, pH 7.0). Ammonium sulfate fractionation was performed as described by Offer *et al.* (30), and the purified myosin was stored at −70 °C under ammonium sulfate.

Chromatography of the myosin by gel filtration on Bio-Gel A-15m, prepared as described by Pollard *et al.* (31), failed to improve purity and caused unacceptable loss of material. The purification of rat myosins was routinely halted after the ammonium sulfate precipitation step.

Enzyme Assays—Purified myosin was resuspended in high salt buffer (0.5 M KCl, 1 mM dithiothreitol, 0.05 M Tris·Cl^-, pH 7.5) and dialyzed overnight against the same buffer to remove ammonium sulfate. After centrifugation at 40,000 × *g* for 15 min to clarify the preparation, the myosin was used for enzymatic analysis.

Potassium-EDTA-activated ATPase was measured in a reaction mixture (0.5 ml) containing 1 mM EDTA, 0.5 M KCl, 5 mM ATP, 50 mM Tris·Cl^-, pH 7.5. Calcium-activated ATPase was measured in a reaction mixture (1.0 ml) containing 10 mM $CaCl_2$, 5 mM ATP, 50 mM Tris·Cl^-, pH 7.5. In each assay, the estimations were performed in triplicate with 0.2 mg of protein/ml of reaction mixture, and controls lacking enzyme were routinely included. The reaction was started by the addition of ATP and incubation was at 25 °C for 10 min. The reaction was stopped by the addition of 0.2 ml of ice-cold 15% (w/v) trichloroacetic acid/ml of reaction mixture. After cooling on ice for 10 min, protein was removed by centrifugation and a 0.3-ml sample of the supernatant was taken for inorganic phosphate determination (32). The results were obtained using assay conditions in which a linear relationship between activity and enzyme concentration was maintained.

Protein Determinations—The fluorescamine method of Bohlen *et al.* (33) was used, with bovine serum albumin as standard.

Gel Electrophoresis—Polyacrylamide gel electrophoresis was carried out in the presence of sodium dodecyl sulfate according to Weber and Osborn (34), using 12.5% acrylamide separating gels or 7.5% cross-linked gels under the electrophoretic conditions of Laemmli (35). Myosin samples were prepared for SDS-polyacrylamide electrophoresis as described by Weeds (7).

Molecular weight of the myosin light chains was determined by comparison with the mobility of marker proteins (aldolase, carbonic anhydrase, trypsin, hemoglobin, and cytochrome *c*) run on 12.5% cross-linked SDS-polyacrylamide gels in phosphate buffer (34).

The subunit composition of myosin light chains was estimated by scanning densitometry (5). The concentration of myosin heavy chains and light chains could not be estimated from densitometric tracings owing to problems encountered when proteins of widely varying molecular weight are analyzed on separate gels (36, 37). Therefore, the percentage of total light chains in myosin was determined by the method of Fenner *et al.* (38). Using this method, both heavy and light chains gave clearly stained bands following electrophoresis on 6% cross-linked SDS-polyacrylamide gels. The bands, stained with Coomassie brilliant blue, were cut out and the dye was eluted by maceration in 25% pyridine in water (v/v). In our hands, the concentration of dye was proportional to absorbance up to 2.2 absorbance units and the absorbance of the eluted dye was proportional to load up to 75 μg of standard protein. The dye elution procedure was applied to myosin extracted, as described above, from each fiber type. The concentration of eluted dye was determined for each sample by running three different myosin concentrations over the linear range for both heavy and light chains using 6% gels. The percentage of total light chains in myosin was thus calculated and the stoichiometry of the light chains was determined using the formula of Lowry and Risby (5), assuming a value of M_r = 470,000 for myosin.

Two-dimensional electrophoresis was performed using the technique of O'Farrell (39). The urea extracts were placed at −70 °C and

electrophoresis was carried out within 2 weeks of sample preparation to minimize storage artifacts.

Polyacrylamide Gel Electrophoresis of Native Myosin—Electrophoresis was carried out at 4 °C in a Pharmacia apparatus (GE-24LS) with recirculation of buffer between the anodic and cathodic chambers. Running buffer was 0.04 M tetrasodium pyrophosphate, pH 8.86, 10% glycerol, 2 mM cysteine. Crude extracts of myosin were prepared using soleus, DVL, SVL, and EDL. The muscles were rapidly dissected, cut into small pieces, and swirled with 3 volumes of Guba-Straub's buffer (0.3 M KCl, 0.1 M KH_2PO_4, 1 mM EDTA, pH 6.5) for 15 min (40). The fragments were pelleted by low speed centrifugation and an equal volume of buffer (80 mM tetrasodium pyrophosphate, pH 8.86) containing 50% glycerol was added to the supernatants. Samples were stored at −20 °C prior to electrophoresis.

Gels (6 × 0.5 cm), 3.2% acrylamide, 0.115% methylene bisacrylamide were prepared in 0.04 M tetrasodium pyrophosphate, pH 8.86, with 0.4% tetramethylethylene diamine, 0.2% ammonium persulfate. Crude muscle extracts, 20–80 μl, were loaded on the gels. Electrophoresis was performed at a constant voltage of 50 V for between 22 and 24 h. ATPase activity in the gels was confirmed using the method described by Hoh *et al.* (41), in which the phosphate released after incubation in substrate was precipitated as calcium phosphate. The myosin isoenzymes were visualized by staining with Coomassie brilliant blue, as described for the regular polyacrylamide gel electrophoresis.

Limited proteolysis of heavy chains and single dimension peptide mapping was accomplished by exploiting the ability of protease to produce reproducible breakdown products from proteins complexed with sodium dodecyl sulfate (42). Purified myosin was electrophoresed on 5% separating gels at 3 mA/gel for 3 h, using a Tris-glycine system in the presence of SDS (35). After rapid staining and destaining for about 15 min, the zones corresponding to the heavy chains were cut from the gels and equilibrated in buffer (0.125 M Tris·HCl, pH 6.8, 0.16% SDS, 1 mM EDTA, 1 mM dithiothreitol). Proteolysis of the heavy chains was accomplished by treating the sample, during electrophoresis, with *Staphylococcus aureus* protease (43). The SDS gel electrophoresis was performed using 7-cm separating and 2-cm stacking gels of 12 and 5% acrylamide concentration, respectively. A gel slice containing about 50 μg of heavy chains was placed on top of the stacking gel and electrophoresis was allowed to proceed at 3 mA/tube for 90 min until the marker dye just entered the stacking gel. *Staphylococcus* protease (10 μl, 0.2 mg/ml of solution in 0.125 M Tris·HCl (pH 6.8), 0.1% SDS, 1 mM EDTA, 20% glycerol) was pipetted onto the top of the stacking gel and the run was continued at the same current for 4.5 h. The gels were stained with Coomassie brilliant blue and destained electrophoretically, and the proteolytic cleavage products were recorded by densitometric scanning using a Gilford recording spectrophotometer.

Physiological Studies—The contractile properties of the fast type IIb SVL, the fast type IIa and IIb EDL, and the slow type I soleus were determined *in vivo* at 22 °C using the isometric and isotonic measuring system described elsewhere (44, 45). A detailed description of the methods used and the experimental results has been published (44, 45). Selected physiological data are included in this paper to enable comparison with the biochemical studies on myosin.

RESULTS

Structural Characterization of Myosin from Rat Skeletal Muscle of Defined Fiber Type—Myosin extracted from SVL (100% type IIb fibers), DVL (70% type IIa fibers), or soleus (85% type I fibers) myofibrils resulted in clearly resolved light chain patterns after SDS electrophoresis.

Slow muscle myosin contains two light chain species and fast white myosin three (Fig. 1, *a* and *c*) in agreement with chick slow and rabbit fast myosin (5, 6). One-dimensional gels resolved four components (LC_{1s}, LC_{1f}, LC_2, and LC_3) in fast red DVL myosin (Fig. 1*b*). However, electrophoresis for longer time resolved two components in the rather broad LC_2 (5,5′-dithiobis(nitrobenzoic acid) light chain) region of the gel, indicating a total of five protein light chain species associated with the DVL sample. The impression that DVL may contain both slow and fast myosin light chains was confirmed by mixing experiments. Equal quantities of purified soleus and DVL myosins separated into a total of four bands upon SDS electrophoresis. These bands correspond to the sum of the contributing light chains, *viz.* LC_{1s}, LC_{1f}, LC_{2s} + LC_{2f}, LC_{3f}, and rat fast (type IIb or IIa) and slow (type I) myosin contain a total of three and two light chain species, respectively.

Molecular Weights, Enzyme Activity, and Stoichiometry of Rat Myosin Light Chains—Analysis of apparent molecular weight of fast twitch red DVL myosin, and a mixture of fast twitch white SVL plus slow twitch soleus myosin, indicates that fast red DVL represents an admixture of fast and slow myosin light chains (Table I). The slow myosin light chains (LC_{1s} and LC_{2s}), observed in the fast red DVL (Figs. 1*b*, 4*c*, and 5*c*), are undoubtably due to the 30% type I fiber contamination of this sample. Further support for this can be found by examining the light chain composition of a fast twitch muscle containing approximately 60% type IIa and 40% type IIb fibers and, importantly, no type I fibers. A two-dimensional analysis of myofibrils from the EDL (Fig. 5*e*) shows three light chains (LC_{1f}, LC_{2f}, and LC_{3f}), a pattern clearly different from the DVL (Fig. 5*c*).

The presence of fast type IIa fibers in rat soleus (24) is

TABLE I

Subunit structure of rat skeletal myosins

Muscles of known fiber type were dissected from normal rat hindlimbs. Myosin was extracted (see "Materials and Methods") from pooled samples from at least 10 animals. Purified myosin was resolved into component light chains by SDS-polyacrylamide gel electrophoresis (34).

Myosin light chains	Fiber type[a]			
	Soleus (type I fibers)	DVL (type IIA fibers)	SVL (type IIB fibers)	Mixed DVL + SVL[b]
		M,		
Alkali 1 (LC_{1s})	27,600 ± 200	27,500 ± 200		27,400 ± 300
(LC_{1f})		25,500 ± 200	25,900 ± 100	25,800 ± 100
DTNB (LC_2)	20,100 ± 200	19,200 ± 200	19,200 ± 100	19,200 ± 200
Alkali 2 (LC_{3f})		15,300 ± 100	15,400 ± 100	15,300 ± 100

[a] Molecular weights were determined by SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn (34) on 12.5% acrylamide gels. The molecular weights of myosin light chains were calculated by comparison with standard proteins run under identical conditions. Values represent the means ± S.E. of eight separate groups.

[b] Myosin, purified from DVL and SVL, was mixed in equal amounts and 50–100 μg of combined myosin protein was loaded on the SDS-polyacrylamide gels.

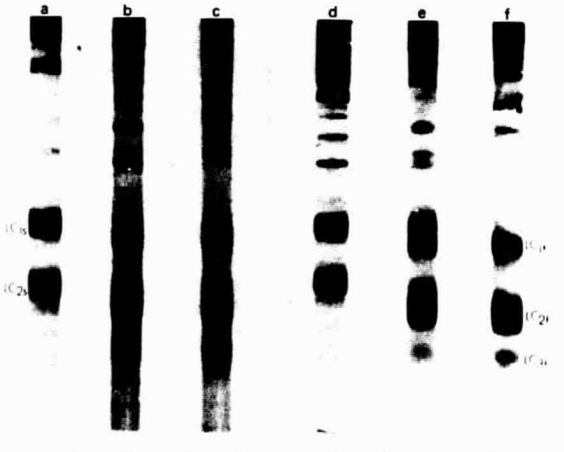

FIG. 1. **One-dimensional electrophoretic comparison of rat skeletal myosins prepared from control and 6-week immobilized rat muscle.** Myosins were prepared as described earlier under "Materials and Methods" and 30–50 μg of protein sample was electrophoresed on 12.5% polyacrylamide gels by sodium dodecyl sulfate. *a*, control soleus; *b*, control DVL; *c*, control SVL; *d*, disuse soleus; *e*, disuse DVL; *f*, disuse SVL.

indicated in heavily loaded two-dimensional gels as two faint spots migrating in the positions of LC_{1f} and LC_{2f} (data not shown).

Stoichiometry of Rat Myosin Light Chains in Various Fiber Types—Percentage of total light chains in myosin was determined by eluting stained heavy and light chain bands from the same gel with pyridine (38) and quantitating the proteins spectrophotometrically (Table II). This method has been used for heart myosin and resulted in 15.5% total light chains in bovine heart (46) and 9.8% total light chains in canine heart (38), compared with 14.7% total light chains in chicken heart, measured by a densitometric method (5). The values we report for the percentage of total light chains in various rat myosins are comparable with those reported for rabbit and chicken (5). Applying the equation of Lowey and Risby (5) and using molecular weights calculated for individual rat myosin light chains (Table I), the stoichiometry of rat myosin light chains was calculated (Table II).

The molecular heterogeneity of rat fast white myosin (SVL) is indicated by the nonequimolar stoichiometry of the alkali light chains, LC_1 being present in twice the molar quantity of LC_3. Rat fast red (DVL) and fast white (SVL) myosin contain approximately equivalent total moles of light chains, in spite of the contamination of DVL with slow red fibers (Figs. 4*c* and 5*c*). Densitometric tracings representative of the myosin light chain distribution in soleus, DVL, and SVL are shown in Fig. 2.

Electrophoresis of Myosin under Nondissociating Conditions—Myosin from rat soleus separated as a single slower migrating component (Fig. 3*a*) compared with the three faster migrating species present in fast twitch myosin (Fig. 3, *b*, *c*, and *d*). Even under conditions of heavy loading, we were unable to demonstrate a faster migrating, minor species, previously reported for rat soleus (40, 47). Myosin from the fast twitch muscles was resolved into three isoenzymatic components FM1, FM2, and FM3, in order of decreasing mobility (48). Mixing experiments demonstrated the separate identity of the slow and fast isomyosins. The three fast isomyosins in type IIb SVL and type IIa/IIb EDL appeared to be identical,

TABLE II

Relative stoichiometry of rat myosin light chains

The moles of light chain in myosin of each fiber type were estimated by the relationship (per cent total light chains in rat myosin) (per cent mass) (molecular weight of myosin)/(molecular weight of light chain) (5).

Light chains[b]	Fiber type[a]					
	Soleus (type I)		DVL (type IIA)		SVL (type IIB)	
	% mass	*mol*	*% mass*	*mol*	*% mass*	*mol*
LC_{1s}	54 ± 2	1.3	16 ± 3	0.3		
LC_{1f}			24 ± 3	0.5	34 ± 2	0.8
LC_2	46 ± 1	1.5	54 ± 2	1.6	60 ± 2	2.1
LC_{3f}			7 ± 1	0.3	9 ± 2	0.4
% total light chains in myosin	14.0 ± 0.2		12.2 ± 0.1		14.2 ± 0.2	

[a] The per cent mass of each myosin light chain was obtained from densitometer traces of SDS gels of myosin from representative fiber types. Each value represents the average of four to six preparations ± S.E., from pooled tissue. The total sum of light chains in each gel is taken as 100%. The moles of light chain were calculated for each myosin-type by the method of Lowey and Risby (5). The values for the percentage of total light chains in myosin were calculated by quantifying the stained heavy and light chains after eluting the dye from 6% polyacrylamide gels (38), and represents an average of seven to nine gels stained with Coomassie brilliant blue at different protein loads for each fiber type.

[b] Molecular weights of light chains were taken from values in Table I.

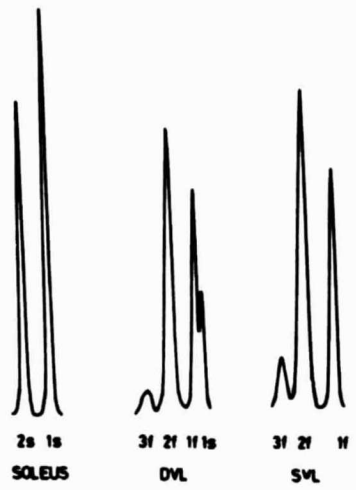

FIG. 2. **Direct copies of densitometer tracings representative of myosin light chain distribution in various rat myosins.** Control myosins were subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Gels were stained with Coomassie brilliant blue and scanned at 590 nm using a Gilford recording spectrophotometer. Protein loading was selected to ensure accurate quantitation of the resolved myosin light chain species.

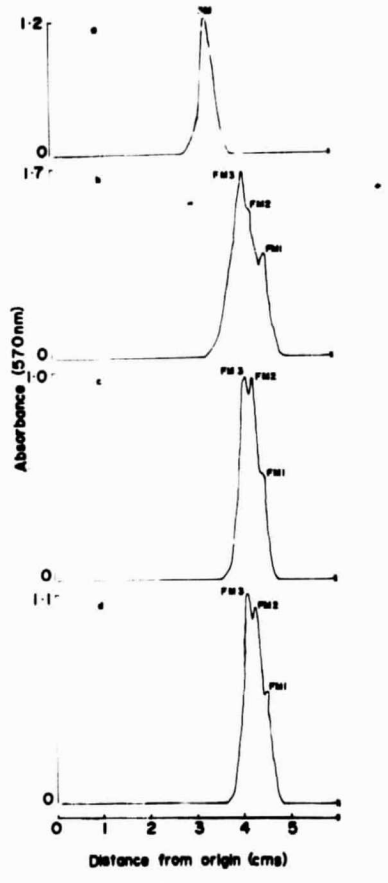

FIG. 3. **Densitometric profiles of rat isomyosins separated by pyrophosphate gel electrophoresis.** Crude muscle extracts (20–80 μl) were analyzed on 3.2% polyacrylamide gels by electrophoresis for 22 h at 4 °C. *a*, slow twitch myosin (*SM*) from rat soleus; *b*, fast twitch myosin components (*FM1, FM2, FM3*) from rat DVL; *c*, fast twitch myosin components (*FM1, FM2, FM3*) from rat SVL; *d*, fast twitch myosin components (*FM1, FM2, FM3*) from rat EDL. The fastest migrating component, FM1, is present in reduced amounts compared with FM2 and FM3 in the rat fast twitch muscles.

since they were superimposable when the crude muscle extracts were mixed prior to electrophoresis. Also, FM3 and FM2 were present in roughly equivalent proportions, with FM1 representing a minor component (Fig. 3, *c* and *d*). Type IIa/I DVL myosin also showed three components, but the slowest migrating FM3 was a broad, heavily staining band in this fast twitch muscle (Fig. 3*b*). The major nature of FM3 was unlikely to be the result of contamination with slow twitch fibers, since mixing soleus and DVL extracts resulted in four isomyosins, SM, FM1, FM2, and FM3, after pyrophosphate electrophoresis (data not shown). The protein species, separated from the crude muscle extracts by pyrophosphate gel electrophoresis, were shown to have ATPase activity and thus to represent isomyosins by the presence of a white precipitate of calcium phosphate of identical mobility to the stained bands following incubation of the gels in substrate (41).

Effect of 6-week Immobilization on Rat Hind Limb Fast Twitch and Slow Twitch Fibers—The calcium-activated and the EDTA-activated myosin ATPase level of the fast twitch DVL and SVL were unaffected by disuse (Table III).

The single (Fig. 1) and double (Fig. 4) dimensional gel patterns for myosin purified from fast twitch muscles were characteristic of the fiber type, but were unaffected by 6 weeks of immobilization. The appearance of additional spots in the electrophoretograms of myofibrillar proteins with identical molecular weights but different isoelectric points (*arrow*, Fig. 5*h*) are probably due to light chain phosphorylation (49). The fast twitch red DVL myosin is clearly resolved into five species of light chain that are unaffected by disuse (Fig. 4, *c* and *d*). The presence of slow twitch light chains in rat DVL, suggested by one-dimensional electrophoresis, is confirmed by resolving mixtures of soleus and DVL myosin. The higher molecular weight LC_1 of rat DVL precisely superimposes upon the larger of the two soleus light chains (Fig. 4*d*). The light chains of fast twitch SVL (Fig. 5*g*) could not be distinguished from those present in fast twitch DVL (Fig. 5*c*) or EDL (Fig. 5*e*).

Immobilization caused a 20% increase in rat soleus calcium-activated myosin ATPase, with no alteration in the distribution or stoichiometry of the myosin light chains. Myosin, purified from control and immobilized rat soleus, was mixed in equal amount and resolved by two-dimensional electrophoresis. The two species of light chain from each sample proved to be superimposable, thereby confirming that soleus responded to disuse by an increase in ATPase unaccompanied by structural changes in the myosin light chains.

The possibility of differential extraction of myosin light chains from the various fiber types was tested by resolving total myofibrillar proteins by two-dimensional electrophoresis. This approach also enabled the effects of disuse to be monitored, with respect to the majority of the contractile proteins (27). The presence of myosin light chains, actin and the two major forms of tropomyosin (alpha-TM and beta-TM) were resolved in each of the myofibrillar preparations (Fig. 5).

Myofibrils of slow twitch soleus (Fig. 5*a*) confirm the presence of the two myosin light chains characteristic of slow twitch fibers. No alteration in the pattern of myofibrillar proteins was observed 6 weeks after immobilization (Fig. 5*b*).

Myofibrils of fast red DVL demonstrate five species of

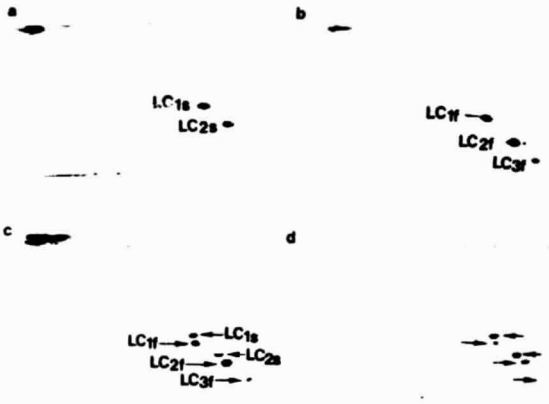

FIG. 4. **Two-dimensional electrophoretic comparison of rat skeletal myosins prepared from soleus (85% type I), DVL (60% type IIa), and SVL (100% type IIb) fibers.** Purified myosins were subjected to isoelectric focusing in the first dimension, followed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Labeling indicates the species of myosin light chain resolved. *a*, soleus; *b*, SVL; *c*, DVL; *d*, soleus mixed with DVL. The myosin light chains are identified, and the *arrows* in *d* represent the same light chains identified in *c*.

TABLE III

Physiological and biochemical properties of rat hind limb muscles

Contractile properties (parameters) measured at 22 °C are according to Witzmann *et al.* (45).

Parameters measured	Fiber type[a]							
	Soleus (85% type I fibers)		DVL (70% type IIA fibers)		EDL (60% type IIA fibers)		SVL (100% type IIB fibers)	
	Control	Immobilized	Control	Immobilized	Control	Immobilized	Control	Immobilized
Contraction time (ms)	130 ± 3	94 ± 4[b]	ND[c]	ND	42 ± 1	47 ± 2[b]	48 ± 1	54 ± 2[b]
One-half relaxation time (ms)	132 ± 4	107 ± 4[b]	ND	ND	28 ± 1	37 ± 3[b]	28 ± 2	36 ± 2[b]
Peak tetanic tension (g/cm^2)	2893 ± 125	1374 ± 146[b]	ND	ND	4392 ± 229	3155 ± 355[b]	1970 ± 97	1918 ± 162
Maximal shortening velocity (mm/s/1000 sarcomeres)	8.3 ± 0.7	13.9 ± 1.9[b]	ND	ND	24.0 ± 0.9	32.4 ± 3.4[b]	22.1 ± 1.1	26.5 ± 1.7[b]
Ca^{2+}-activated myosin ATPase activity (μmol·mg^{-1}·min^{-1})	0.342 ± 0.013	0.414 ± 0.020[b]	0.734 ± 0.021	0.683 ± 0.061	ND	ND	0.955 ± 0.035	0.956 ± 0.025
K^+-EDTA-activated myosin ATPase activity ATPase activity (μmol·mg^{-1}·min^{-1})	0.405 ± 0.046	0.457 ± 0.039	1.085 ± 0.117	0.989 ± 0.150	ND	ND	1.105 ± 0.068	1.095 ± 0.079

[a] Values are means ± S.E. $n = 10$ for physiological observations, and $n = 4$ for myosin data.

[b] Immobilized significantly different from control, $p < 0.05$.

[c] ND, not determined.

myosin light chains, thereby confirming the presence of slow twitch fibers in the DVL muscle. The contamination with slow twitch light chain is not observed in either fast twitch EDL (Fig. 5*e*) or fast twitch SVL (Fig. 5*g*).

None of the myofibrillar preparations demonstrated structural alterations in any contractile proteins following immobilization.

Myosin Heavy Chains—Since immobilization resulted in an increased *soleus* myosin ATPase activity, but identical light chain pattern, compared with normal slow red muscle, it was of interest to determine if these changes were associated with alteration in the heavy chain primary structure (43, 50). As a first step toward analyzing molecular alterations, the myosin heavy chains were subjected to digestion by *S. aureus* protease in the presence of SDS. The proteolytic cleavage products of the entire myosin heavy chain were analyzed by one-dimensional gel electrophoresis. Densitometric scans of the proteolytic pattern of heavy chains indicate unambiguous and reproducible differences between slow twitch red soleus and fast twitch white SVL (Fig. 6, *a* and *b*). The pattern

FIG. 5. **Two-dimensional electrophoretic comparison of rat skeletal myofibrillar proteins prepared from control and 6-week immobilized rat muscle.** Washed myofibrils were prepared from muscles of selected fiber type and solubilized in isoelectric focusing buffer. The apparent molecular weights of rat myosin light chains (*LC*) are given in Table I and the values for other myofibrillar proteins are: α-tropomyosin (*TM*), M_r = 32,000; βTM, M_r = 36,000; α-actin, M_r = 43,000. The myosin heavy chain region of each gel is not shown. *a*, control soleus; *b*, immobilized soleus; *c*, control DVL; *d*, immobilized DVL; *e*, control EDL; *f*, disuse EDL; *g*, control SVL; *h*, disuse SVL. The *arrow* illustrates the double spot resulting from possible phosphorylation of SVL LC_{2f}. The acidic end (pH 5) of the first dimension isoelectric focusing gel is to the *right*, the basic end (pH 8) to the *left*.

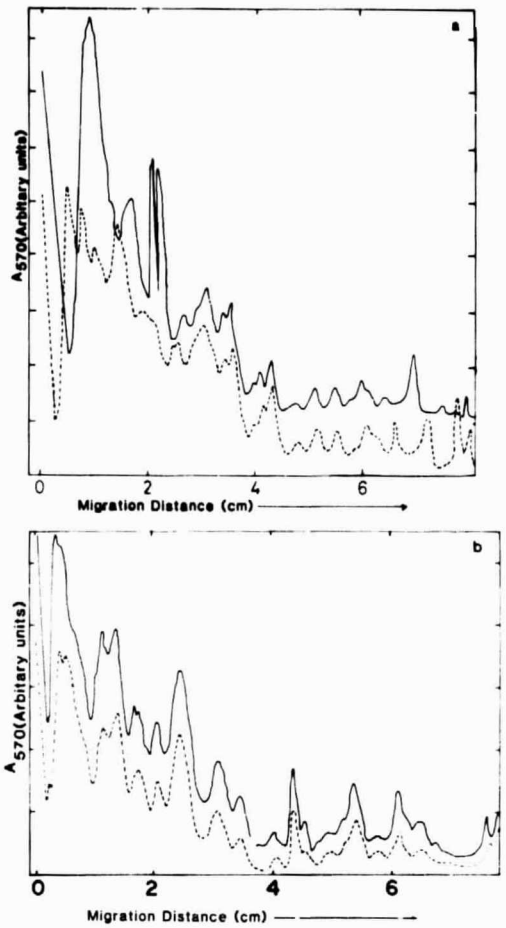

FIG. 6. **Densitometric profiles of gel patterns of peptides produced by limited proteolysis of rat myosin heavy chains.** Each scan represents the distribution of peptides in 12% polyacrylamide gels produced by proteolysis of about 50 μg of heavy chain from either control or 6-week immobilized rats. The densitometric profiles of the immobilized muscles is represented by a *broken line* which is vertically displaced along the absorbance axis to enable comparison with control, *solid line*, samples. *a*, slow twitch soleus heavy chain peptide scan; *b*, fast twitch SVL heavy chain peptide scan.

obtained after proteolysis of fast twitch red DVL myosin proved to be more complex and variable from run to run (data not shown), presumably reflecting both admixture of fast twitch and slow twitch fibers in this muscle and structural differences between fast red and fast white myosin heavy chains (24, 61). In agreement with previous enzyme measurement and light chain analysis, the proteolytic cleavage patterns of fast white myosin extracted from normal animals was indistinguishable, within experimental precision, from patterns from 6-week immobilized rats (Fig. 6*b*). The densitometric profiles of immobilized and control soleus myosin heavy chain peptides (Fig. 6*a*) displayed clear differences, particularly in peptides of high molecular weight.

Although the Ca^{2+}-activated myosin ATPase activity was increased by immobilization in slow but not fast twitch muscles, all muscles showed an elevated maximal shortening velocity (Table III). The per cent increase was considerably higher in the soleus (67% increase) than in the predominantly type IIa EDL (35% increase) or the 100% type IIb SVL (20% increase). The isometric twitch duration (contraction time and one-half relaxation time) was prolonged by disuse in the fast EDL and SVL, while inactivity shortened the twitch duration of the slow soleus.

DISCUSSION

Muscle fiber structure satisfies two main functional requirements, speed of contraction, which is correlated with myosin ATPase activity (51) and is higher in fast white fibers than in slow red, and force generation. Fast twitch type IIa and type IIb fibers appear to be indistinguishable by physiological criteria; rat EDL and SVL have similar contraction times, one-half relaxation times, and maximal shortening velocities (Table III). The myosin ATPase-specific activities of fast red and fast white fibers can be calculated from the data in Table III. If we accept that rat soleus muscle contains 85% type I fibers and 15% type IIa fibers (24), and we assume that the specific activity of type IIa myosin ATPase is the same as type IIb, then the specific activity of type I myosin ATPase may be calculated as 0.234 μmol of $P \cdot mg^{-1} \cdot min^{-1}$ (using values in Table III) from the relationship: per cent type IIa fibers × specific activity of type IIa ATPase + per cent type I fibers × specific activity of type I fibers = measured soleus Ca^{2+}-ATPase specific activity. Similar logic applies to the DVL. The measured activity of 0.734 agrees well with the theoretical calculated value, 0.7(0.955) + 0.3(0.234) = 0.739. The concept that there is no detectable difference between myosin ATPase activities of fast red and fast white fibers receives further support from the observation (Table IV) that rat EDL, comprising 60% type IIa and 40% type IIb fibers, has a myosin ATPase activity comparable with rat SVL (Table III), which is comprised of 100% type IIb fibers. The maximal shortening velocity of rat slow red soleus is about one-third of the corresponding values for fast white SVL and mixed fast red/fast white EDL (Table III), and this physiological parameter of muscle function correlates well with the Ca^{2+}-activated myosin ATPase activities of these fiber types. However, the relationship between fiber typing and myosin ATPase activity is not straightforward. The soleus of cat and guinea pig is considered pure slow red muscle homogeneous for type I fibers on the basis of both histochemical fiber typing (24, 59) and the distribution of mitochondria (60), while rabbit and rat soleus are rated inhomogeneous slow twitch muscles (59, 60).

TABLE IV

Comparison of Ca^{2+}-activated myosin ATPase activities (at 25 °C), expressed in micromoles of $P_i \cdot mg^{-1} \cdot min^{-1}$, for fast twitch and slow twitch muscles of various species

The data are compiled from studies using myosin purified from identified fiber types.

Muscle	Species	Ca^{2+}-activated ATPase	Reference
Soleus	Rabbit	0.14	Syrovy & Gutmann (52)
	Rabbit	0.19	Syrovy & Gutmann (53)
	Guinea pig	0.22	Syrovy & Gutmann (52)
	Rabbit	0.24	Sreter *et al.* (54)
	Rat	0.35	Gutmann & Syrovy (55)
	Rat	0.34	Present study
	Cat	0.36	Van Winkle *et al.* (56)
	Rat	0.42	Syrovy & Gutmann (52)
	Rat	0.43	Gutmann & Syrovy (55)
EDL	Rabbit	0.58	Syrovy & Gutmann (52)
	Rabbit	0.60	Syrovy & Gutmann (53)
	Rabbit	0.84	Sreter *et al.* (54)
	Rabbit	0.88	Sreter *et al.* (57)
	Rat	0.90	Syrovy & Gutmann (52)
	Rat	0.86	Gutmann & Syrovy (55)
	Guinea pig	0.89	Syrovy & Gutmann (52)
Tibialis anterior (white region)	Cat	1.01	Van Winkle *et al.* (56)
Tibialis anterior	Rabbit	0.90	Sreter *et al.* (57)
SVL	Rabbit	1.0	Sarkar *et al.* (58)
	Rat	0.96	Present study
DVL	Rat	0.73	Present study
Tibialis anterior	Cat	0.76	Van Winkle *et al.* (56)

However, the histochemically homogeneous cat soleus has a myosin ATPase activity more comparable with the mixed fiber rat soleus than with the relatively pure slow twitch rabbit soleus (Table IV). This disparity in myosin ATPase activities in the slow twitch soleus from various species cannot be reconciled with the myosin light chain pattern, since LC_1 is a doublet in the cat and the rabbit (7, 56), but only a single polypeptide in the rat (21) and guinea pig (61).

The light chain stoichiometry of rat soleus myosin, calculated in the present study, corresponds to values published for rat (62) and rabbit (63). The presence of LC_{3f} was not normally detected in myosin purified from rat slow twitch soleus muscle. However, rat soleus myofibrils stored in the freezer for 5 months prior to myosin extraction revealed a protein species that migrated on Laemmli-type SDS gels with a mobility identical with LC_{3f}. Paradoxically, this component was not present in immobilized rat soleus myofibrils similarly stored. It would appear that this band is more likely to represent a storage artifact, than myosin of the fast type phenotype (64).

Rat fast twitch white SVL and fast twitch red DVL muscles cannot be distinguished on the basis of myosin ATPase or the distribution and molecular weights of the light chains. The relative abundance of fast twitch light chains (Table II) in the rat SVL compares with other mammalian fast twitch muscles (5, 61, 62), except for the low level of LC_{3f} in the rat. It is not clear if this represents a true decrease in alkali 2 chains in the rat or an extraction problem, since it was only by examining total cellular proteins that Keller and Emerson (65) were able to demonstrate the presence of LC_{3f} in an embryonic system. Quantitation from two-dimensional gels of total myofibrillar protein extracts would be needed to resolve this question. However, the distribution of light chains of fast muscle can be correlated with the proportion of individual isoenzymatic species. The individual fast isomyosins have been shown to differ in light chain composition, *viz.* FM1 comprises $(LC_2f)_2(LC_3f)_2$, FM2 is $LC_1f(LC_2f)_2$ LC_3f, and FM_3 corresponds to $(LC_1f)_2$ LC_2f (40, 48, 50). The fact that the isomyosin profile of rat SVL shows equivalent proportions of FM2 and FM3, with FM1 present as a minor component, would correlate with the small proportion of LCf_3 in this muscle. Rat SVL myosin is similar to rabbit fast myosin (66, 67), containing 2 mol of LC_2/mol and LC_1 in 2-fold molar excess over LC_3, but the alkali light chains are present in 1.6 times molar excess in the rabbit compared with the rat. The reduced content of essential light chains in rat compared with rabbit SVL does not affect the myosin ATPase activity (Table IV). Rat fast twitch red myosin differs from rat fast twitch white myosin in light chain stoichiometry and in the percentage of total light chains, and these features may be reflected in the differential staining of these fiber types by immunofluorescent antibodies prepared against the alkali light chains (68).

Hind limb immobilization failed to alter either the light chain distribution or stoichiometry in any of the fiber types examined. However, the maximal shortening velocity was significantly increased after 6 weeks of disuse in both the slow twitch and the fast twitch muscles (Table III), but only in the immobilized soleus was there an increased myosin ATPase activity. Clearly, elevated ATPase activity and increased mechanical V_{max} is accomplished without alteration in the myosin light chain pattern. Recent work on skinned fibers has demonstrated no correlation between V_{max} and the ratio of the myosin light chains in fast twitch or slow twitch fibers (69). Furthermore, although fibers from fast twitch and slow twitch muscles could clearly be distinguished on the basis of their light chain patterns and V_{max}, the relative proportions of the light chains present were independent of tension development, muscle stiffness, or unloaded shortening velocity (69).

The myosin from different types of muscle can be distinguished on the basis of the pattern of peptides generated by proteolytic digestion of the heavy chains. This statement is true for both slow twitch and fast twitch muscles of the chicken (43), and in this manner, guinea pig fast twitch red massater was distinguished from fast twitch white tensor fasciae latae (61). This latter distinction could not be made unambiguously in the present study, presumably because rat DVL is an admixture of both slow red and fast red fibers (24).

Further work is needed to determine the sites and extent of changes in amino acid sequence that may be brought about in the rat soleus myosin heavy chain by short term immobilization. Although short term immobilization of rat slow twitch soleus resulted in increased V_{max}, higher ATPase activity, and altered pattern of heavy chain proteolytic cleavage products, long term immobilization can cause the synthesis of fast type light chains in cat soleus (22), suggesting that the response to disuse may be temporally related. The increased V_{max} in fast twitch SVL and EDL suggests that mechanical measurement offers a more sensitive indicator of alteration in muscle function with short term immobilization than does biochemical analysis.

Acknowledgments—We would like to thank Dr. Marion Greaser for his helpful suggestions on the purification of myosin, and Duyen Tran for technical assistance. We are grateful to Barbara DeNoyer for typing the manuscript.

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